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Neurofilament proteins NF-L, NF-M and NF-H in brain of patients with Down syndrome and Alzheimer's disease

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Summary. Neurofilaments (NFs) are integral constituents of the neuron playing a major role in brain development, maintenance, regeneration and the pattern of expression for NFs suggests their contribution to plasticity of the neuronal cytoskeleton and creating and maintaining neuronal architecture. Using immune-histochemical techniques the altered expression of NFs in Down syndrome (DS) and Alzheimer's disease (AD) has been already published but as no corresponding systematic immune-chemical study has been reported yet, we decided to determine proteins levels of three NFs in several brain regions of DS and AD brain.

We evaluated immunoreactive NF-H, NF-M and NF-L levels using Western blotting in brain regions temporal, occipital cortex and thalamus of patients with DS (n = 9), AD (n = 9) and controls (n = 12). We found significantly increased NF-H in temporal cortex (controls: means 0.74 ± 0.39 SD; DS: means 3.01 ± 2.18 SD) of DS patients and a significant decrease of NF-L in occipital cortex of DS and AD patients (controls: means 1.19 ± 0.86 SD; DS: means 0.35 ± 0.20; AD: 0.20 ± 0.11 SD).

We propose that the increase of NF-H in temporal cortex of DS brain is due to neuritic sprouting as observed in immune-histochemical studies. The increase may not be caused by the known accumulation of NFs in plaques, tangles or Lewy bodies due to our solubilization protocol. The decrease of NF-L in occipital cortex of DS and AD patients may well be reflecting neuronal loss. Altogether, however, we suggest that NFs are not reliable markers for neuronal death, a hallmark of both neurodegenerative diseases, in DS or AD. The increase of NF-H in DS or the decrease of NF-L in DS and AD leaves the other NFs unchanged, which points to dysregulation in DS and AD and raises the question of impaired structural assembly of neurofilaments.

Keywords: Amino acids – Down syndrome – Alzheimer's disease – Neurofilaments – NF-L – NF-M – NF-H

Introduction

Neurofilaments (NFs) i.e. intermediate filaments of the nervous system make up the bulk of axonal volume in large myelinated fibres and appear as solid, rope-like fibrils of 8–12 nm in diameter ranging from tens to even hundreds of micrometers' length (Hammerschlag et al., 1993). The primary type of intermediate neurofilaments (class IV) in neurons is formed from three subunit polypeptides, the NF triplet, that were initially identified from axonal transport studies. The apparent molecular weights for the NF subunits vary widely across species, but mammalian forms typically range from 180–200 kDa for the high molecular weight subunit (NF-H), 130–170 kDa for the middle subunit (NF-M) and 60–70 kDa for the low molecular weight subunit (NF-L).

NF subunits are distinct polypeptides each coded by a separate gene (Fliegner and Liem, 1991). NFs play a critical role in determining axonal calibre and have characteristic side arms, unique among intermediate filaments, which are formed by NF-M and NF-H carboxyl terminal regions. NF-M and NF-H have unusually high levels of phosphorylation. Neuronal populations vary in their number of NFs, the polypeptide composition of those NFs and the degree of phosphorylation (Brady, 1992). Both, expression and phosphorylation of the NF triplet are regulated during development, maintenance and regeneration and the pattern of expression for NFs suggests their contribution to plasticity of the neuronal cytoskeleton and creating and maintaining neuronal architecture.

Most publications described NF changes in Down syndrome (DS) and Alzheimer's disease (AD) using immune-histological techniques. In AD, Dickson and co-workers (1999) found that an abnormal accumulation of variably phosphorylated NFs represented the earliest cytoskeletal alteration associated with dystrophic neurite formation. Nakamura and co-workers (1997) reported the abnormal distribution of NF-L in neurons with AD: while a polyclonal antibody against NF-L did not stain control brains, in brain with early onset type of AD labelled many neurons and dystrophic neurites. Vickers and co-workers (1994) described the distribution of NF triplet class proteins in hippocampus of AD patients aged patients and controls. They demonstrated that in aged hippocampus increased NF-immunoreactivity in CA1 was observed; in AD surviving neurons in CA1 showed intense labelling of NFs with many of the neurons giving off "abnormal" sprouting processes. The authors also carried out immunoblotting and found that there was a proportionally greater amount of NFs in hippocampal tissue of AD patients.

A series of publications focused on the neuropathological relevance for NFs with the consistent finding, that immune-histochemically NFs can be found in senile plaques (Cras et al., 1991; Schmidt et al., 1991; Su et al., 1996; Su et al., 1998), tangles (Zhang et al., 1989) and Lewy bodies (Doering, 1993; Trojanowsky et al., 1993; Pollanen et al., 1994) of patients with AD.

In brain of patients with DS de la Monte and co-workers (1996) detected increased age associated neurite NF-immunoreactivity in Layers I and II of the cerebral cortex beginning at 1 year of age, followed by positive neurofibrillary tangles beginning at age 5 years and NF-immunoreactivity

plaques beginning in the third decade. Patients with DS develop AD neuropathological changes from the fourth decade and NF changes comparable to those seen in AD can be therefore expected.

The aim of our study was to quantify NF proteins NF-L, NF-M and NF-H in several brain regions of adult patients with DS and AD neuropathological changes and as no (immune-chemical) quantification of these NFs was carried out in these neurodegenerative disorders and immune-histochemical studies from several groups were either not comparable or did not reveal a consistent NF-pattern, we studied NF expression by an immunoblotting method.

Materials and methods

Brain samples

The brain regions temporal, occipital and thalamus of patients with DS (n = 9; 3 females, 6 males; 55.6 ± 7.4 years old), AD (n = 9; 2 females, 7 males; 76.8 ± 5.6 years old) and controls (n = 12; 9 females, 3 males; 47.3 ± 18.6 years old), used and characterised in a previous publication (Labudova et al., 1998), were used for the studies in the protein level. Briefly, post-mortem brain samples were obtained from the MRC London Brain Bank for Neurodegenerative Diseases, Institute of Psychiatry. In all DS brains there was evidence of abundant beta A plaques and neurofibrillary tangles. The AD patients fulfilled the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) criteria for probable AD (Tierney et al., 1998). The histological diagnosis of AD was established and consistent with the CERAD criteria (Mirra et al., 1991) for a "definitive" diagnosis of AD. The controls were brains from individuals with no history of neurological or psychiatric illness. The major cause of death was bronchopneumonia in DS and AD patients and heart disease in controls. Post-mortem interval of brain dissection in AD, DS and controls was 24.6 ± 21.7, 31.4 ± 17.5 and 33.0 ± 15.1 hours. Tissue samples were stored at −70°C and the freezing chain was never interrupted.

Western blotting

Shock frozen brains of patients with DS, AD and controls were thawed on ice in the presence of the protease inhibitor Pantinol^{R} (Gerot, Austria) 500 KIE/ml homogenization solution and mixed 1:5 (w/v) with homogenization solution (0.25 M sucrose, 1 mM EDTA, 3 mM imidazole, 0.1% ethanol, pH 7.2). Samples were homogenized for 30 seconds (six strokes) at 440 re/min in a Potter-Elvejhjem homogenizer on ice. The homogenate was centrifuged for 5 minutes at 3,000 g and 4°C. The supernatant was used for the determination of proteins (Labudova et al., 1998) and immunoblotting. Protein concentration of the brain homogenates were determined by the BCA protein assay kit (Pierce, USA). The sample buffer (125.5 mM Tris base, 70 mM sodium dodecyl sulphate (SDS), 0.001% Bromphenol blue, 20% glycerol, 2% 2-mercaptoethanol, pH 6.8) was added in the same volume of the supernatants containig from 10 to 15 μg of proteins. The samples were then heated at 37°C for 5 minutes.

The proteins were separated electrophoretically on 7.5% (NFs) and 12.5% (beta-actin) homogeneous gels (Amersham Pharmacia Biotech AB, Sweden) using Multiphor II Electrophoresis System (Amersham Pharmacia Biotech AB, Sweden) according to the recommendations of manufacturer. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes by Multiphor II NovaBlot Unit (Amersham Pharmacia Biotech AB, Sweden) at the transfer condition – 0.8 mA per cm^2 of a membrane for 1 hour. Membranes (Immobilon™-P, MilliPore, USA) were washed 2

times for 5 minutes in Tris buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and blocked in the blocking solution (100 mM Tris-HCl, 150 mM NaCl, pH 7.5, 1 mM $MgCl_2$, 0.1% Tween-20, 1.5% non-fat dry milk (Bio-Rad, USA)) at 4°C overnight. After the blocking the membranes were probed with the polyclonal rabbit antineurofilament H 200 kD (Serotec, United Kingdom), polyclonal rabbit antineurofilament M 160 kD (Serotec, United Kingdom), polyclonal rabbit antineurofilament L 70 kD (Serotec, United Kingdom) diluted 1:5000 (v/v), and with monoclonal anti-actin antibody N350 (Amersham Pharmacia Biotech AB, Sweden) diluted 1:3000 in the blocking solution for 75 minutes (NFs) and 60 minutes (beta-actin) at room temperature. The membranes were washed three times for 15 minutes in the blocking solution prior to incubation at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, USA) diluted 1:5000 (v/v) (NFs) and goat anti-mouse Ig (Southern Biotechnology Associates, USA) diluted 1:3000 (v/v) (beta-actin) in the blocking solution. The membranes were washed again three times for 15 minutes and the blots were developed with the Western blot chemiluminiscence reagents (NEN™ Life Science Products, Inc., USA).

Densitometry and statistics

Developed films were scanned and densities of the NFs and beta-actin immunoreactive bands on the films were calculated by RFLP Scan2.1 software program (Scanalytics, USA). Between group differences were calculated by non-parametric Mann-Whitney U-test. The level of significance was considered at $P < 0.05$.

Results

The Western blot patterns allowed fair assignment of bands to the apparent molecular weights for the individual NF-proteins at 70 kDa for NF-L, 147 kDa for NF-M and 185 kDa for NF-H. A series of NF-immunoreactive bands of lower molecular weights, possibly representing truncated forms or cleavage products were not quantified. Evaluation of total NF-L, NF-M or NF-H-immunoreactivity (quantification of all bands) led to enormous variation even in the control group and we therefore decided to use the band with the corresponding assigned apparent molecular weights for the individual NFs. No major individual band possibly representing cleavage product or truncated was regularly observed and therefore no quantification of these bands was performed, taking into account that we are thus missing information on degradation of NFs. The numerical results are presented in the Tables 1 and 2.

NF-H levels were significantly increased in DS temporal cortex and this finding was also observed when NF-H was related to the housekeeping gene beta-actin. NF-M levels did not differ between the groups in the individual brains regions and corresponding findings were obtained when related to beta-actin. NF-L was decreased in occipital cortex of AD and also the NF-L/beta-actin ratio was decreased in this region. NF-L and NF-L/beta-actin are also decreased in occipital cortex of DS.

Discussion

As shown in the results, NF-H and the ratio of NF-H/beta-actin was significantly increased in temporal cortex of patients with DS, whereas NF-M

Table 1. Neurofilament levels (arbitrary units) in temporal cortex, occipital cortex and thalamus

NF	Area	Control	DS	AD
NF-H	Temporal cortex	0.74 ± 0.39 (n = 7)	3.01± 2.18* (n = 7)	0.94 ± 0.77 (n = 7)
	Occipital cortex	0.30 ± 0.19 (n = 8)	0.40 ± 0.36 (n = 5)	0.57 ± 0.29 (n = 8)
	Thalamus	4.62 ± 3.43 (n = 11)	2.75 ± 1.43 (n = 6)	2.64 ± 1.44 (n = 6)
NF-M	Temporal cortex	1.60 ± 1.50 (n = 7)	1.02 ± 0.50 (n = 7)	1.66 ± 1.40 (n = 5)
	Occipital cortex	2.39 ± 1.46 (n = 8)	1.35 ± 1.07 (n = 6)	1.10 ± 0.43 (n = 6)
	Thalamus	0.46 ± 0.32 (n = 9)	0.25 ± 0.04 (n = 3)	0.46 ± 0.46 (n = 5)
NF-L	Temporal cortex	0.23 ± 0.21 (n = 8)	0.31 ± 0.13 (n = 7)	1.37 ± 1.37 (n = 6)
	Occipital cortex	1.19 ± 0.86 (n = 8)	0.35 ± 0.20* (n = 5)	0.17 ± 0.13** (n = 5)
	Thalamus	0.31 ± 0.20 (n = 11)	0.21 ± 0.17 (n = 4)	0.15 ± 0.11 (n = 6)

Optical density, arbitrary units in mean ± SD. Significant difference (* $P < 0.05$; ** $P < 0.01$) compared to control.

Table 2. Neurofilament levels normalized versus beta-actin (arbitrary units) in temporal cortex, occipital cortex and thalamus

NF	Area	Control	DS	AD
NF-H	Temporal cortex	0.81 ± 0.57 (n = 7)	3.05 ± 2.23* (n = 7)	1.21 ± 1.04 (n = 7)
	Occipital cortex	0.27 ± 0.17 (n = 8)	0.47 ± 0.43 (n = 5)	0.62 ± 0.28 (n = 8)
	Thalamus	2.50 ± 2.04 (n = 11)	3.14 ± 1.56 (n = 6)	2.99 ± 1.43 (n = 6)
NF-M	Temporal cortex	1.65 ± 1.43 (n = 7)	1.08 ± 0.61 (n = 7)	2.49 ± 2.43 (n = 5)
	Occipital cortex	2.15 ± 1.38 (n = 8)	1.59 ± 1.40 (n = 6)	1.12 ± 0.52 (n = 6)
	Thalamus	0.23 ± 0.16 (n = 9)	0.38 ± 0.29 (n = 3)	0.43 ± 0.25 (n = 5)
NF-L	Temporal cortex	0.23 ± 0.19 (n = 8)	0.35 ± 0.24 (n = 7)	1.71 ± 1.51 (n = 6)
	Occipital cortex	1.11 ± 0.96 (n = 8)	0.37 ± 0.25* (n = 5)	0.15 ± 0.11** (n = 5)
	Thalamus	0.16 ± 0.12 (n = 11)	0.20 ± 0.12 (n = 4)	0.24 ± 0.14 (n = 6)

Values are the mean ± SD of optical density. Significant difference (* $P < 0.05$; ** $P < 0.01$) compared to normal controls.

levels were not altered in any brain region of DS. A significant and remarkable decrease of NF-L and NF-L/beta-actin was observed in occipital cortex of DS patients, however. In AD, NF-L and the ratio NF-L/beta-actin were significantly decreased in occipital cortex. Our findings of increased NF-H and increased NF-H after normalization versus the housekeeping gene beta-actin are in agreement with already reported immune-histochemical findings: In the only systematic NF-study on DS brain, de la Monte and co-workers (1996) detected increased age associated neurite NF-immunoreactivity in Layers I and II of the cerebral cortex beginning at 1 year of age, followed by positive neurofibrillary tangles beginning at age 5 years and NF-immunoreactivity in plaques in the third decade. We did not find any significant changes of NF-M in DS cortical or subcortical regions; but NF-L and NF-L related to beta-actin, showed a decrease in DS occipital cortex.

We have to see the results in the light of the solubilization procedure: we used a protocol which would not have been solubilizing plaques, tangles or Lewy bodies, regularly containing NFs (see above, introduction). NFs are considered as neuronal markers and as neuronal death and cell loss is a hallmark of DS (Cairns, 1999) we would have been expecting decreased NF levels, which holds for NF-L in occipital cortex, a brain region regularly affected in both neurodegenerative disorders. Neuritic sprouting in DS brain (de la Monte et al., 1996) may have been compensating a tentative decreased NF-L in other regions thus probably masking neuronal cell loss.

Furthermore, elevated NF levels when normalized versus beta-actin have to be interpreted with care: Actins are death substrates in apoptosis, which unequivocally takes places in DS. In our study no significant differences were found between the three groups studied in temporal and occipital lobe. Only in thalamus we detected significant decrease of beta-actin levels in Down syndrome and Alzheimer' disease patients in comparison to controls. One has also to take into account that significant glial proliferation occurs in DS-brain (Greber et al., 1999) thus affecting brain actin levels. The use of other housekeeping-genes as e.g. enzymes from glucose metabolism cannot be used in DS as well; glucose metabolism is regularly and seriously disturbed in DS brain and therefore we do the interpretation of results basically without normalization versus a housekeeping gene (Labudova et al., 1999).

It is of particular interest that the NF expressional pattern was inconsistent which could suggest different regulation of the pathophysiological process taking place in DS pathobiology or point to non-uniform distribution and association of triplet proteins in neurofilaments as already proposed by Nash and Carden (1995). Also speculative one could assume that the immune-histochemical finding of sprouting (see above) would involve mainly this isoform which in turn raises the question whether the increased NF-H reflects disturbed assembly of NFs or would be disturbing the assembly of NFs by changed ratios.

In AD brain a significant decrease of NF-L in occipital cortex (which holds when normalized versus beta-actin) with normal NF-M levels was detected, resembling findings in DS, which is plausible as neuropathological findings are largely comparable in adult DS and AD with all DS patients aged $>$ 40 years

(and so does our cohort of DS patients) presenting with Alzheimer brain pathology (Cairns, 1999). As stated in the introduction most reports showed increased NFs by immune-histochemical techniques (see above) but we were unable to confirm these observations using our immune-chemical technique. Again, we have to state that using our solubilization technique, we did not determine accumulating/aggregating NFs in plaques, tangles or Lewy bodies.

Our data are confirming decreased mRNA levels for NF-L by Kittur (1994) and others found decreased mRNA for NF-L in hippocampal CA1 (Sommerville et al., 1991), or decreased NF-L in parietal cortex (Clark et al., 1989). Our findings of unchanged NF-M would be compatible with a report from Chow and co-workers (1998) who studied single neurons from AD brain; mRNA for NF-M was found to be unchanged in neocortical isolated neurons confirming in situ hybridization studies. A major factor that the individual reports are not comparable is not only simply technological in nature but also due to the lack of information on the state of phosphorylation of the NFs. This is of particular importance when immune-chemical studies are employed.

In conclusion, we report that NF-H is elevated in DS temporal cortex whereas in occipital cortex NF-L was decreased, which may indicate different regulation of NFs and neuronal loss in the case of NF-L. The underlying cause for this increase seems to be neuritic sprouting as seen on histology. Decreased NF-L in occipital cortex of AD in turn may well reflect neuronal loss.

We may draw the conclusion that NFs cannot be used as reliable markers for neuronal loss in DS and AD.

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